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ABSTRACT Equine arteritis virus (EAV) has the ability to establish persistent infection in the reproductive tract of the stallion (carrier) and is continuously shed in its semen. We have recently demonstrated that EAV persists within stromal cells and a subset of lymphocytes in the stallion accessory sex glands in the presence of a significant local inflammatory response. In the present study, we demonstrated that EAV elicits a mucosal antibody response in the reproductive tract during persistent infection with homing of plasma cells into accessory sex glands. The EAV-specific immunoglobulin isotypes in seminal plasma included IgA, IgG1, IgG3/5, and IgG4/7. Interestingly, seminal plasma IgG1 and IgG4/7 possessed virus-neutralizing activity, while seminal plasma IgA and IgG3/5 did not. However, virus-neutralizing IgG1 and IgG4/7 in seminal plasma were not effective in preventing viral infectivity. In addition, the serological response was primarily mediated by virus-specific IgM and IgG1, while virus-specific serum IgA, IgG3/5, IgG4/7, and IgG6 isotype responses were not detected. This is the first report characterizing the immunoglobulin isotypes in equine serum and seminal plasma in response to EAV infection. The findings presented herein suggest that while a broader immunoglobulin isotype diversity is elicited in seminal plasma, EAV has the ability to persist in the reproductive tract, in spite of local mucosal antibody and inflammatory responses. This study provides further evidence that EAV employs complex immune evasion mechanisms during persistence in the reproductive tract that warrant further investigation.

KEYWORDS equine arteritis virus, equine viral arteritis, EAV, EVA, immunoglobulin isotype, humoral response, seminal plasma, mucosal immunity, neutralization, immune evasion, persistent infection, reproductive tract

Equine arteritis virus (EAV) is a small, enveloped virus with a positive-sense, single-stranded RNA genome (+ssRNA; the genome is ~12,704 bp and contains at least 10 open reading frames [ORFs]) (1–4) and belongs to the family *Arteriviridae* (genus *Equartevirus*), order *Nidovirales*, a group that includes porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), lactate dehydrogenase-elevating virus of mice (LDV), and the recently recognized wobbly possum disease virus (WPDV) of free-ranging Australian brushtail possums (*Trichosurus vulpecula*) in New Zealand (1, 4–8). EAV is the causative agent of equine viral arteritis (EVA), a respiratory and reproductive disease of equids (1, 5, 9–11).

While the vast majority of natural infections are inapparent, occasional outbreaks of EVA occur and are clinically characterized by influenza-like clinical signs in adult horses,

abortion in pregnant mares, and fatal bronchointerstitial pneumonia or pneumoenteric syndrome in foals (12). Most importantly, EAV can establish persistent infection in the reproductive tract of 10 to 70% of infected stallions (carrier state) (13–18). Persistently infected stallions continuously shed EAV solely in their semen without exhibiting any clinical signs or a loss of fertility (1, 5, 11–14, 16, 19, 20). The carrier state is testosterone dependent and can last from several weeks or months (viral shedding for <1 year postinfection; short-term carriers) to several years or even lifelong (viral shedding for >1 year postinfection; long-term carriers), despite the development of a strong neutralizing antibody response in serum (1, 5, 11–15, 20, 21). Recently, it has been demonstrated that host genetics are implicated in the outcome of infection (i.e., the short- or long-term carrier state) in the stallion (22, 23). Carrier stallions constitute the natural reservoir for EAV and, thus, play a key epidemiological role, as they ensure the maintenance and perpetuation of the virus in equine populations between breeding seasons (1, 11, 12). Moreover, they constitute a source of viral genetic and antigenic variability that can lead to the emergence of novel variants of the virus (19, 24–27; B. Nam, Z. Mekuria, M. Carossino, G. Li, Y. Zheng, J. Zhang, R. F. Cook, J. R. Campos, K. M. Shuck, E. Bailey, P. J. Timoney, and U. B. R. Balasuriya, unpublished data).

The EAV particle consists of a nucleocapsid core and seven envelope proteins. GP5 (~20 to 44 kDa) and M (~17 kDa) are the major envelope proteins of the virus, and they form a disulfide-linked heterodimer in the particle. Viral glycoprotein GP5 contains four major neutralization sites (namely, sites A, B, C, and D) that have been extensively characterized both genetically and antigenically by the use of neutralizing monoclonal antibodies (MAbs) (28–31). GP5 and M protein heterodimerization is critical for the posttranslational modification (glycosylation) and conformational maturation of the neutralization determinants in GP5 and for the induction of antibody responses (28–34). Even though considerable variation in the amino acid sequence of the GP5 glycoprotein is observed, there is only one known serotype of EAV, and most field viral strains are broadly neutralized using high-titer polyclonal equine antisera raised against the prototype virulent Bucyrus strain (VBS) of EAV (32, 35).

Previous studies have demonstrated that after infection EAV induces both specific complement-fixing (CF) and virus-neutralizing (VN) antibodies (36, 37), which provide long-lasting immunity and protection from reinfection with most (if not all) viral strains. Both CF and VN antibodies develop between 7 and 14 days postinfection (dpi), and their levels peak at either 2 to 3 weeks or 2 to 4 months postinfection, respectively. Complement-fixing antibodies decline rapidly, while VN antibodies persist for 3 years or more (15, 17, 32, 34, 36). Both structural proteins (GP5, M, and N) and nonstructural proteins (nsp2, nsp4, nsp5, and nsp12) induce humoral immunity (38, 39).

The appearance of serum neutralizing antibodies coincides with the successful neutralization and clearance of EAV from the bloodstream and all body tissues by approximately 28 dpi (35). However, EAV persists in the reproductive tract of carrier stallions regardless of the high titers of neutralizing antibodies present in the serum of these animals (15, 40). The pathogenesis of persistent EAV infection, including the host factors associated with the establishment and maintenance of persistent infection, is not fully comprehended and is under extensive investigation in U. B. R. Balasuriya's laboratory. Thus, to better understand the EAV-host interaction in the reproductive tract and the viral mechanisms of immune evasion at the site of persistent infection, characterization of the host mucosal antibody response to EAV during persistence is of utmost importance. We hypothesized that EAV escapes antibody-mediated neutralization in the reproductive tract and, consequently, continues to be shed in the semen of infected stallions. In this study, we characterized the mucosal antibody isotype response to EAV during persistent infection and identified the presence of EAV-specific neutralizing immunoglobulins in seminal plasma. The findings from this study further demonstrate that EAV employs a complex mechanism of immune evasion during persistent infection in the reproductive tract, with successful viral escape from systemic humoral and mucosal antibody responses, as well as local inflammatory responses.
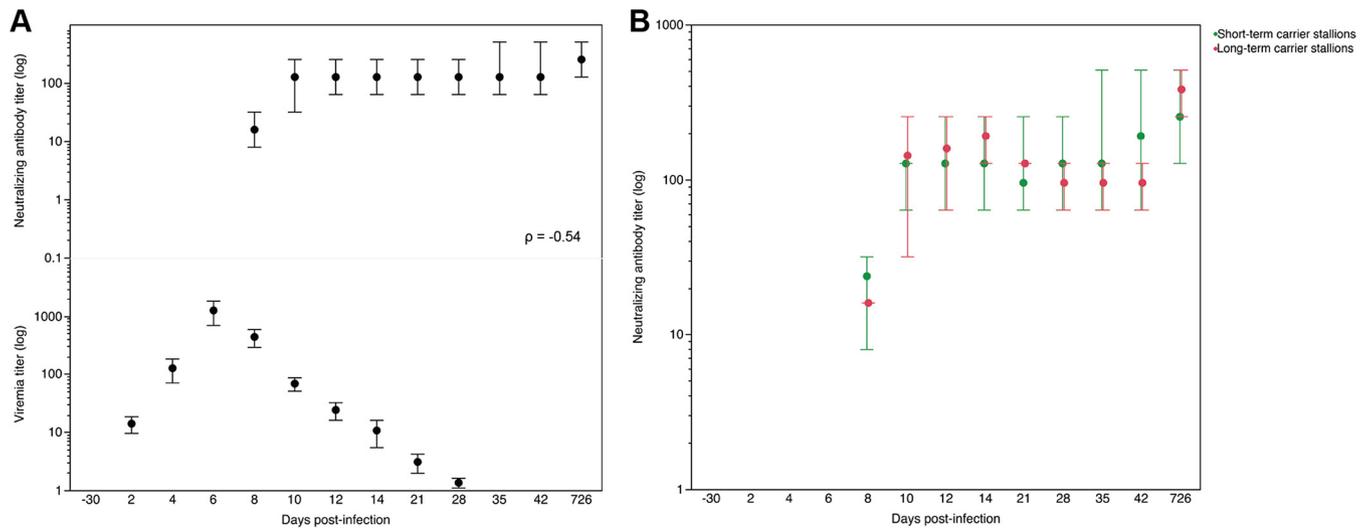
FIG 1 Neutralizing antibody and viremia titers following experimental EAV infection of stallions ($n = 8$). A negative correlation between the rise in neutralizing antibody titers and the viremia titers was observed (A), but no differences in neutralizing antibody titers between short-term ($n = 6$) and long-term ($n = 2$) persistently infected stallions were detected (B).

RESULTS

Clinical outcome and establishment of persistent infection in stallions experimentally infected with EAV. The clinical outcome after intranasal challenge with the EAV KY84 strain in the group of stallions evaluated in this study has been described in other studies (40, 41). Briefly, all experimentally infected stallions ($n = 8$) developed moderate to severe clinical signs of EVA following experimental infection, which lasted a median of 20 days (interquartile range [IQR] = 11.25 days), and no signs of disease or reproductive dysfunction were observed after clinical recovery. Viral shedding in semen started at between 3 and 5 dpi, and 5/8 experimentally infected stallions (stallions L137, L138, L139, L141, and L142) shed EAV in semen for a median of 165 days (IQR = 263.5 days) and were classified as short-term carrier stallions (approximate duration of shedding, ≤1 year), while 2/8 stallions (stallions L136 and L140) continued to shed EAV in their semen for ≥726 dpi (i.e., long-term carrier stallions' shedding duration, >1 year). For behavioral reasons, semen collection from one of the stallions (stallion L143) was not feasible, and the carrier status could not be determined until the end of the study. The absence of infective virus, viral antigen, and RNA from tissues derived from the reproductive tract after euthanasia and the minimal inflammation observed in the accessory sex glands were indicative that this animal stopped shedding earlier in the course of the study and was considered to be a short-term carrier stallion (40).

Characterization of serum neutralizing antibody response during EAV persistent infection in stallions. Neutralizing antibodies in serum were initially detected by 8 dpi in all experimentally infected stallions ($n = 8$), with a median antibody titer of 1:16 (range, 1:8 to 1:32). Significantly higher neutralizing antibody titers were observed by 10 dpi, with a median titer of 1:128 (range, 1:32 to 1:256; $P < 0.001$) (Fig. 1A). Serum neutralizing antibody titers steadily increased and remained high until the end of the study (726 dpi) independently of the infection status of the stallion (i.e., long-term or short-term carrier). The median neutralizing antibody titer by 726 dpi was 1:256 (range, 1:128 to 1:512). The increase in serum neutralizing antibody titers showed a moderate and significant negative correlation with viremia titers ($\rho = -0.54$), suggesting that the development of neutralizing antibodies might be associated with the clearance of EAV from the bloodstream and other body tissues, with the exception of the reproductive tract (Fig. 1A). Differences in antibody titers between long-term ($n = 2$) and short-term ($n = 6$) carrier stallions were not observed at any time point ($P > 0.05$) (Fig. 1B).
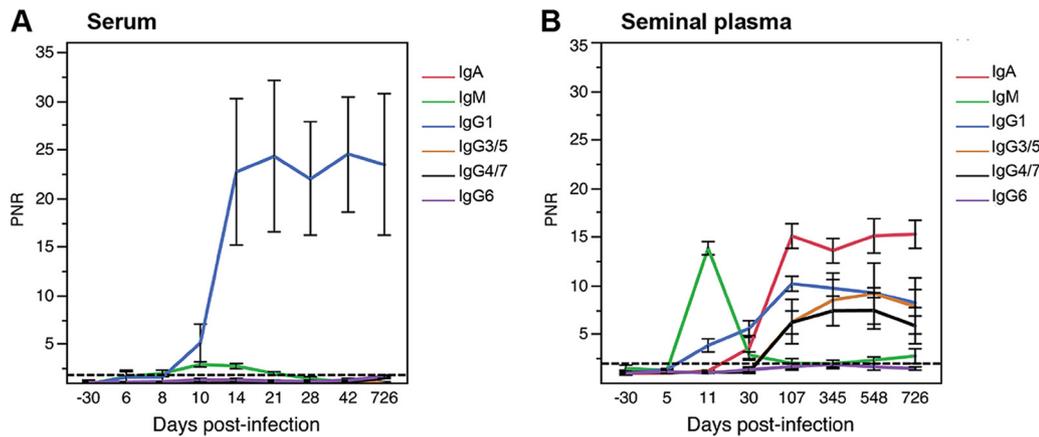
FIG 2 Serological (A) and seminal plasma (B) antibody isotype responses following experimental EAV infection of stallions ($n = 8$ and $n = 7$, respectively). (A) Serological antibody responses were mediated by an early virus-specific IgM response and a later onset of virus-specific IgG1. (B) Seminal plasma antibody responses were mediated by several antibody isotypes, including virus-specific IgA, IgM, IgG1, IgG3/5, and IgG4/7. The mean positive/negative ratios (PNRs) and standard errors of the means (bars) are represented. The PNR cutoff value for all isotypes is represented by the dashed lines (the PNR cutoff was 2 for all ELISAs, with the exception of the seminal plasma IgM ELISA, for which the PNR cutoff was 3).

Characterization of serological immunoglobulin isotype profile during EAV persistent infection in stallions. The immunoglobulin profile induced by EAV in the serum of experimentally infected stallions was characterized using a panel of EAV-specific capture isotyping enzyme-linked immunosorbent assays (ELISAs) (IgM, IgA, IgG1, IgG3/5, IgG4/7, and IgG6). The humoral response to EAV was characterized by an early onset of virus-specific IgM (6 to 8 dpi) (Fig. 2A), which was coincident with seroconversion, as determined by a virus neutralization test (VNT). Only two out of eight stallions showed a positive IgM response as early as 6 dpi (stallions L136 and L143). Fifty percent of the animals had detectable EAV-specific IgM by 8 dpi (stallions L136, L139, L142, and L143), and 100% (8/8) had a strong response by 10 dpi. The IgM-mediated response peaked at between 10 and 14 dpi (mean positive/negative ratio [PNR] = 2.95, $P < 0.05$) and rapidly decayed by 28 dpi (mean PNR < 1.5) (Fig. 2A) in both short- and long-term carrier stallions. An isotype switch to EAV-specific IgG1 followed the initial IgM response, with an onset at 10 dpi (mean PNR = 5.16) in 5/8 stallions, coincident with a significant rise in neutralizing antibody titers. EAV-specific IgG1 responses were significantly increased by 14 dpi ($P < 0.01$) (Fig. 2A). Interestingly, one stallion (stallion L137) showed a very early IgG1 response (6 dpi, PNR = 5.3). The EAV-specific IgG1 response peaked by 21 dpi (mean PNR = 24.3) and remained high in the serum (mean PNR > 20, with individual variations in responses) (Fig. 2A) of experimentally infected stallions. The IgG1-specific response strongly correlated with the neutralizing antibody titers ($\rho = 0.62$), and no statistically significant differences in the magnitude of either the IgM or the IgG1 response between short-term ($n = 2$) and long-term ($n = 6$) carrier stallions were observed (Fig. 3A). Finally, virus-specific IgA, IgG3/5, IgG4/7, or IgG6 isotype responses were not detected in the serum of experimentally infected stallions during the course of the study (PNRs < 2) (Fig. 2A and 3A).

Characterization of seminal antibody responses during EAV persistent infection in stallions. Detection of EAV-specific seminal antibodies in sequential seminal plasma samples collected from a group of experimentally infected stallions ($n = 7$) was performed by capture isotyping ELISAs, as described in Materials and Methods. The seminal immunoglobulin profile was characterized by several EAV-specific immunoglobulin isotypes, including IgM, IgA, and IgG, with several subtypes being involved (IgG1, IgG3/5, and IgG4/7) (Fig. 2B). EAV-specific seminal plasma IgM followed a pattern similar to that for serum IgM, with a peak of shedding occurring at 11 dpi (mean PNR = 13.86, $P < 0.0001$) (Fig. 2B). Even though 4/7 stallions presented IgM levels above the baseline level by 30 dpi (mean PNR = 3.5), the levels decayed thereafter (Fig. 3B). No
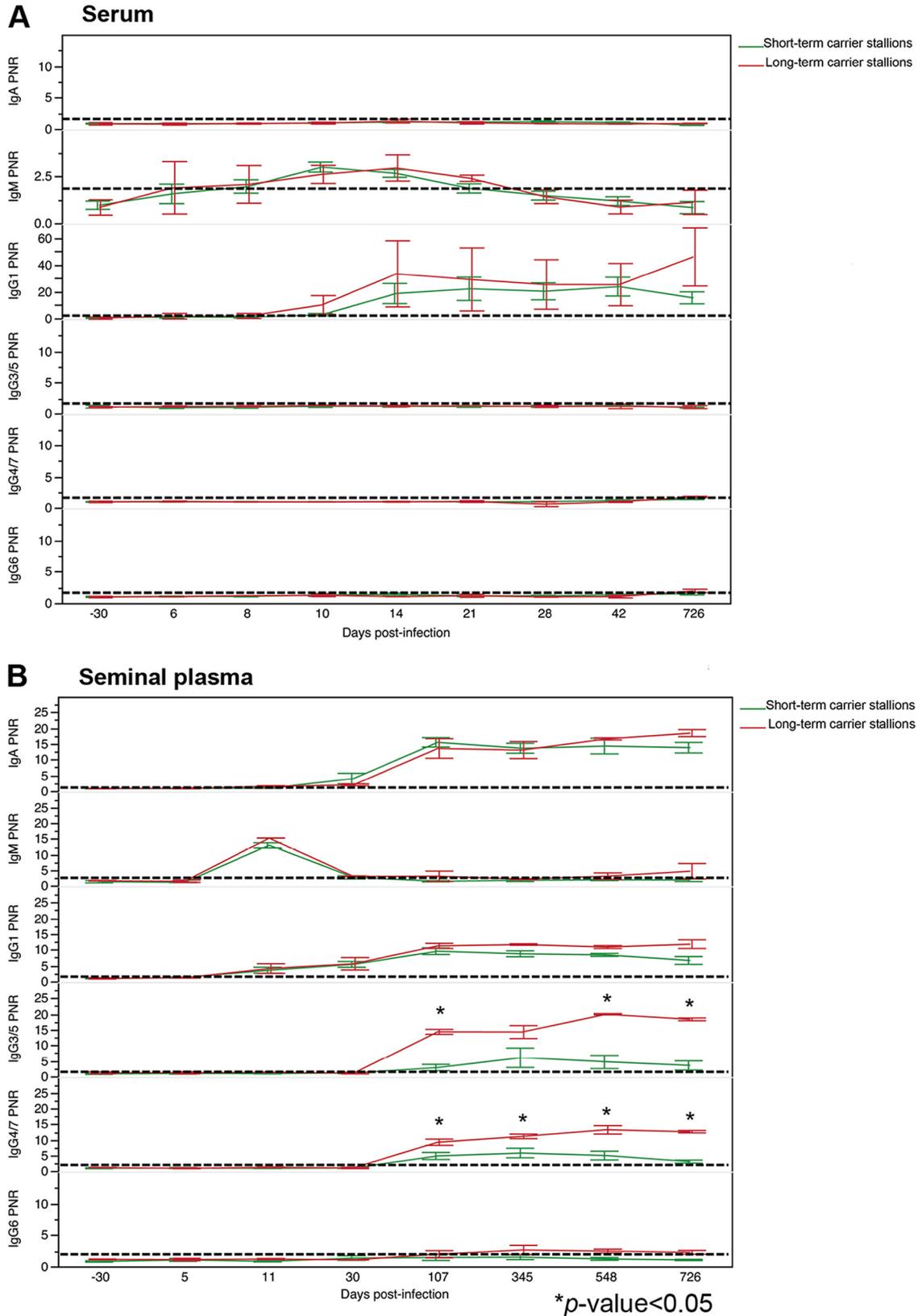
FIG 3 Serological (A) and seminal plasma (B) antibody isotype responses during short-term ($n = 6$) and long-term ($n = 2$) persistent infection in stallions following experimental EAV infection. The mean positive/negative ratios (PNRs) and standard errors of the means (bars) are represented. The PNR cutoff value for all isotypes is represented by the dashed lines (the PNR cutoff was 2 for all ELISAs, with the exception of the seminal plasma IgM ELISA, for which the PNR cutoff was 3).

statistically significant differences between short- and long-term carrier stallions were observed (Fig. 3B). Furthermore, EAV-specific IgA secretion was initially detected in seminal plasma by 30 dpi (mean PNR = 3.6) (Fig. 2B) in 5/7 stallions and peaked by 107 dpi (mean PNR = 15.13) ($P < 0.0001$). IgA levels remained high until the conclusion of the study (726 dpi) (Fig. 2B), and no significant differences between short- and long-term carrier stallions were observed at any time point (Fig. 3B).

The EAV-specific seminal immunoglobulin profile included the IgG1, IgG3/5, and IgG4/7 subtypes. The seminal plasma IgG1 profile followed a pattern similar to that observed for serum IgG1, and IgG1 became detectable in seminal plasma by 11 dpi (mean PNR = 3.9), reaching its peak by 107 dpi (mean PNR = 10.23) ($P < 0.001$). The seminal plasma IgG1 level remained high until the end of the study, and significant differences in the magnitude of its response were not observed between short- and long-term carrier stallions (Fig. 3B). The EAV-specific mucosal IgG3/5 and IgG4/7 responses were triggered later in the course of infection. In the case of seminal plasma IgG3/5, it was detectable by 107 dpi and peaked by 548 dpi (mean PNR = 9.21, $P < 0.05$) (Fig. 2B). Interestingly, long-term carrier stallions presented significantly higher PNR values for the seminal IgG3/5 response than short-term carrier stallions, specifically, at 107, 548, and 726 dpi ($P < 0.01$) (Fig. 3B). Seminal plasma IgG3/5 remained detectable until the end of the study (726 dpi), except in one stallion (stallion L139, a short-term carrier). Two out of five short-term carrier stallions did not have detectable IgG3/5 in seminal plasma at any time point (stallions L137 and L138). Seminal plasma IgG4/7 was detected by 107 dpi (7/7 stallions, $P < 0.01$), and peak concentrations were observed by 548 dpi (mean PNR = 7.5) and remained detectable until 726 dpi (Fig. 2B), with significantly higher PNR values being seen in long-term carrier stallions than short-term carrier stallions from 107 dpi until the conclusion of the study ($P < 0.05$) (Fig. 3B). EAV-specific IgG6 subtype responses were not detected in the seminal plasma of either long- or short-term carrier stallions during the course of the study.

Specificity of seminal immunoglobulin isotypes for EAV major envelope (GP5 and M) and nucleocapsid proteins. Serum and seminal plasma (mucosal) immunoglobulin specificity was evaluated by Western immunoblotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of viral proteins from gradient-purified EAV. Serum and seminal plasma anti-EAV IgG1, as well as seminal plasma anti-EAV IgA and IgG4/7, presented strong binding to viral proteins of 20 to 44 kDa, 17 kDa, and 14 kDa, corresponding to EAV major structural proteins GP5 and M and the nucleocapsid (N) protein, respectively (Fig. 4). Seminal plasma anti-EAV IgG3/5 was demonstrated to be specific for the viral GP5 glycoprotein and N protein, while its specificity for the M protein could not be determined (Fig. 4). The faint bands observed at 30 kDa (GP5) and 14 kDa (N) could be associated with lower concentrations of this subtype in seminal plasma or a lower affinity to EAV structural proteins.

Neutralizing capacity of virus-specific mucosal antibody isotypes derived from stallions experimentally infected with EAV. Serum IgG1 and seminal plasma IgA, IgG1, IgG3/5, and IgG4/7 were purified from pooled serum and seminal plasma samples collected at 726 dpi to evaluate their ability to neutralize EAV. A modified microneutralization assay demonstrated that anti-EAV IgG1 and IgG4/7 in seminal plasma elicit virus neutralization under *in vitro* conditions. The IgG1 and IgG4/7 purified from the seminal plasma of short- and long-term carrier stallions presented similar neutralization endpoints (i.e., the difference was <4-fold). The IgG1 neutralization endpoint in seminal plasma was estimated to be at average of 6 μg/ml and 3 μg/ml of purified IgG1 for short- and long-term carrier stallions, respectively, while the IgG4/7 neutralization endpoint in seminal plasma was estimated to be 2 μg/ml of purified IgG4/7 for both groups. These neutralization endpoints were similar to those determined for purified serum IgG1 (3 μg/ml). In contrast, IgG3/5 and IgA purified from short- and long-term carrier stallions did not neutralize EAV under *in vitro* conditions even at concentrations of ≥25 μg/ml.

Mucosal antibody production in the accessory sex glands during EAV persistent infection in stallions. EAV infection induced an inflammatory response in the
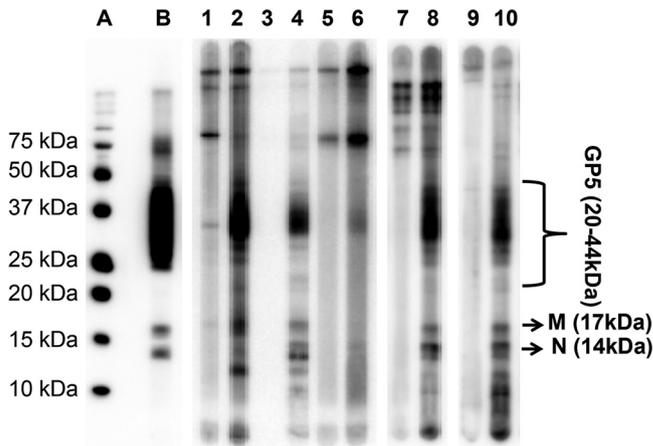
FIG 4 Specificity of serum and seminal plasma immunoglobulin isotypes for EAV major structural (GP5 and M) and nucleocapsid (N) proteins during persistent infection in stallions. Serum IgG1 and seminal plasma IgG1, IgG4/7, and IgA demonstrated strong reactivity with viral GP5, M, and N, while IgG3/5 presented weak reactivity with viral GP5 and N. Lanes: A, molecular mass markers; B, control equine arteritis virus GP5-, M-, and N-specific monoclonal/polyclonal antibodies; 1, prechallenge serum IgG1; 2, serum IgG1 obtained at 726 dpi; 3, prechallenge seminal plasma IgG1; 4, seminal plasma IgG1 obtained at 726 dpi; 5, prechallenge seminal plasma IgG3/5; 6, seminal plasma IgG3/5 obtained at 726 dpi; 7, prechallenge seminal plasma IgG4/7; 8, seminal plasma IgG4/7 obtained at 726 dpi; 9, prechallenge seminal plasma IgA; 10, seminal plasma IgA obtained at 726 dpi. Lanes A and B are representative controls (molecular mass markers and purified EAV, respectively). The results presented in lanes 1 to 6, 7 and 8, and 9 and 10 were obtained in independent experiments.

reproductive tract of experimentally infected stallions, and an extensive characterization of the inflammatory infiltration has been previously described (40). Histological lesions in the accessory sex glands of experimentally infected stallions that were actively shedding EAV at the time of euthanasia ($n = 2$ long-term carrier stallions) included a bilateral, moderate, multifocal lymphoplasmacytic ampullitis (Fig. 5A and B), while experimentally infected stallions that underwent viral clearance and interrupted viral shedding by the time of euthanasia presented with minimal, focal, lymphoplasmacytic ampullitis ($n = 6$; short-term carrier stallions). Even though inflammatory lesions were infrequent in vesicular glands and the prostate, a mild lymphoplasmacytic adenitis of the bulbourethral glands with occasional mononuclear cell foci was observed (Fig. 5C and D). Inflammatory lesions in the accessory sex glands of control (naive) stallions ($n = 4$) frequently ranged from being absent to being present as minimal and focal lymphoplasmacytic infiltrates, with these lesions showing a significantly lower severity and distribution and significantly lower cumulative scores than those for long-term carrier stallions ($P < 0.01$), while no statistically significant differences between naive and short-term carrier stallions were observed, as previously shown (40). In addition, specific immunostaining scores for diverse inflammatory cells infiltrating the ampullae were demonstrated to be significantly higher in long-term carrier stallions than short-term carrier and control stallions ($P < 0.0001$) (data not shown) (40). Interestingly, the bulbourethral glands were abundantly infiltrated with plasma cells, as observed by hematoxylin and eosin (H&E) staining and confirmed by specific immunostaining, as described below (Fig. 5 to 7), including in naive stallions.

Specific immunoglobulin-producing plasma cell populations in the accessory sex glands of experimentally infected ($n = 8$) and naive ($n = 4$) stallions were analyzed by immunohistochemistry (IHC). Immunoglobulin-specific plasma cells were identified throughout the male accessory sex glands and included IgA-positive (IgA+), IgG1-positive (IgG1+), IgG3/5-positive (IgG3/5+), and IgG4/7-positive (IgG4/7+) plasma cells (Fig. 6 and 7). IgA+ plasma cells were the most abundant plasma cell type distributed along the different male accessory sex glands, particularly in the bulbourethral glands (with scores of 4 in all stallions evaluated, $P < 0.01$) and ampullae (median scores, 3.5
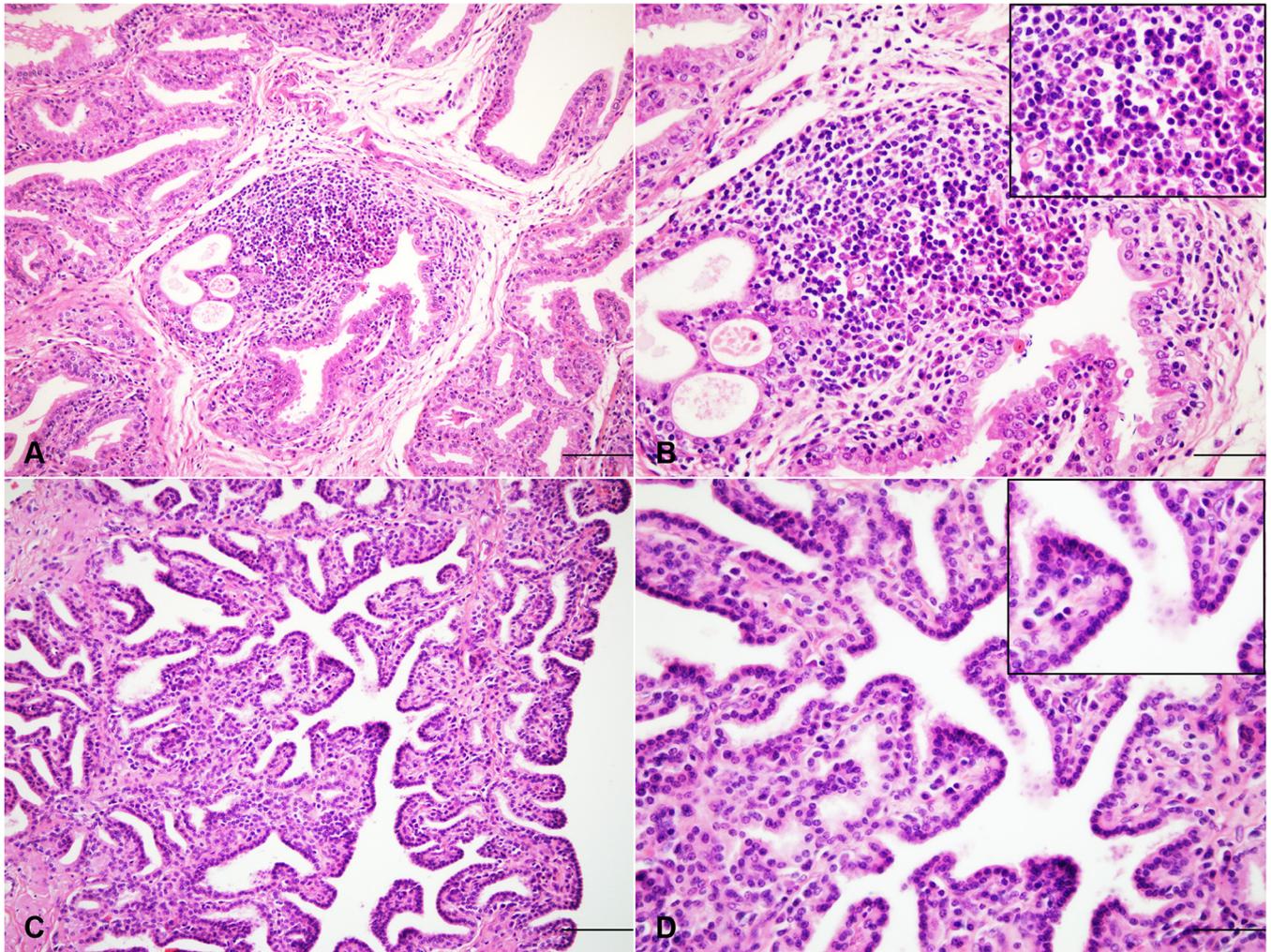
FIG 5 Inflammatory infiltration within accessory sex glands of stallions persistently infected with EAV. (A) Lymphoplasmacytic ampullitis. H&E stain. Magnification, ×200. Bar, 100 μm. (B) Higher magnification of the inflammatory focus shown in panel A. Inflammatory cells included lymphocytes and plasma cells (inset) in multifocal aggregates and lymphocytes and plasma cells dispersed in the lamina propria. H&E stain. Magnification, ×400. Bar, 50 μm. (C) Lymphoplasmacytic adenitis, bulbourethral gland. H&E stain. Magnification, ×200. Bar, 100 μm. (D) Higher magnification of the inflammatory cells shown in panel C. Inflammatory cells included lymphocytes and plasma cells (inset) in occasional foci and lymphocytes and plasma cells, particularly plasma cells, dispersed within the lamina propria,. H&E stain. Magnification, ×400. Bar, 50 μm.

and 2 in long- and short-term carrier stallions, respectively) (Fig. 6 and 7A and B). The IgA$^+$ plasma cell distribution was rare to absent in the ampullae of naive stallions (median score, 0.5), while IgA$^+$ plasma cells were as abundant in other glands of naive stallions as in those of infected stallions. Moreover, scattered IgA$^+$ glandular epithelial cells were frequently observed in all accessory sex glands (ampullae, vesicular glands, prostate, and bulbourethral glands) (Fig. 8A and B) and presented intense cytoplasmic, apical, and/or basolateral staining. Epithelial staining demonstrated the presence of mucosal, transepithelial IgA transport to the glandular lumen. In addition, homing of IgG1$^+$ plasma cells primarily involved the ampullae of long-term carrier stallions (median score, 3) and short-term carrier stallions (median score, 1) and the bulbourethral gland of short-term carrier stallions (median score, 2) (Fig. 6 and 7C and D), while their presence was rare in most of the accessory sex glands of naive stallions (median score, 0.5); the exception was the bulbourethral glands (median score, 2). IgG3/5$^+$ plasma cells were broadly distributed in the accessory sex glands of long-term carrier stallions, while low numbers were detected in tissues from short-term carrier stallions (Fig. 6 and 7E and F) and rarely in the ampullae, vesicular glands, and prostate from naive stallions (median scores, 0 to 0.5). Finally, IgG4/7$^+$ plasma cells were observed in
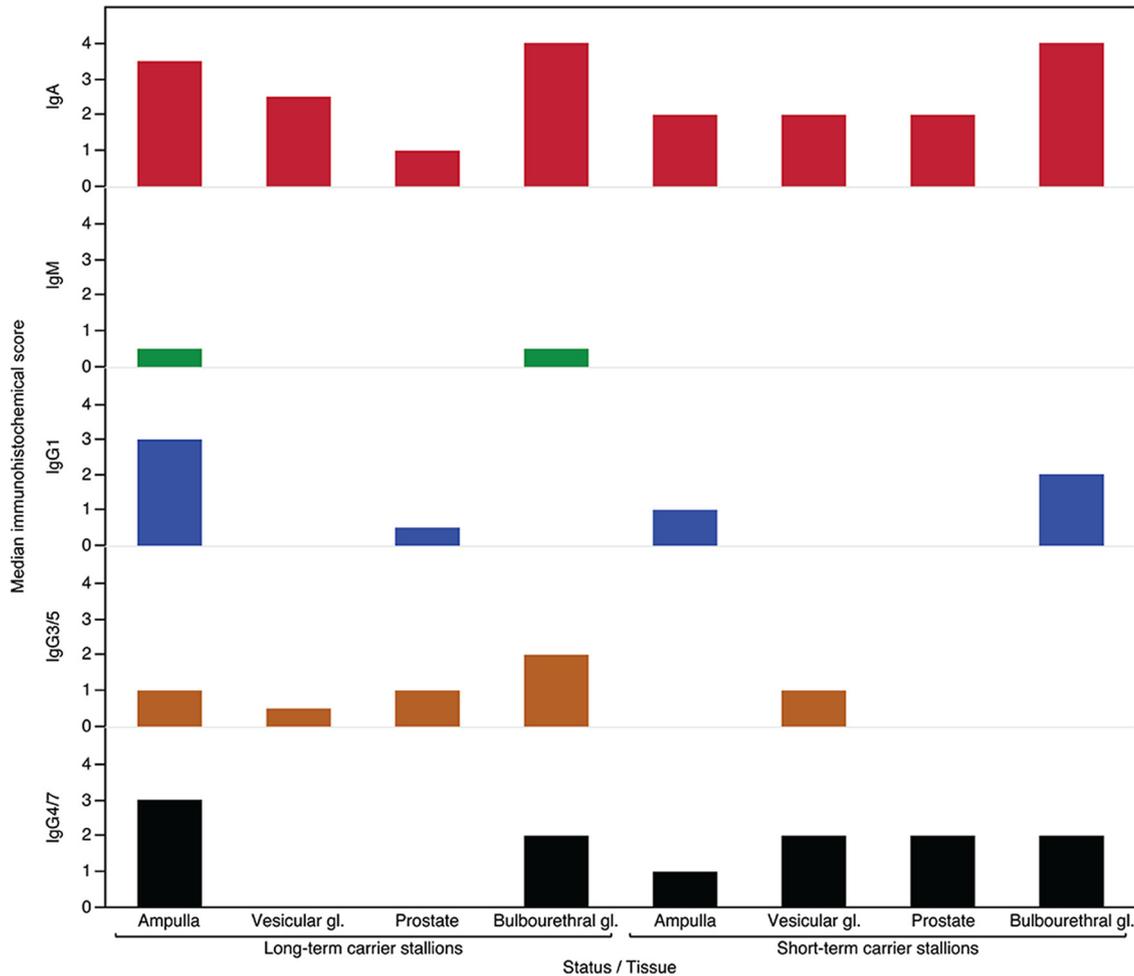
FIG 6 Distribution of immunoglobulin-specific plasma cells within accessory sex glands (gl.) during EAV persistent infection in stallions. Plasma cells presented a predominant homing to the ampullae and bulbourethral glands in long-term carrier stallions.

large numbers in the ampullae and bulbourethral glands of long-term carrier stallions, while they were observed in all accessory sex glands of short-term carrier stallions (Fig. 6 and 7G and H). Naive stallions presented with occasional IgG4/7$^+$ plasma cells in vesicular glands and the prostate (median score, 1), but, interestingly, higher numbers of these and IgG3/5$^+$ plasma cells were observed in the bulbourethral glands. No statistically significant differences in plasma cell abundance (as determined by IHC scores) in accessory sex gland tissues, with the exception of the ampullae (see below), from long-term carrier, short-term carrier, or naive stallions were observed ($P > 0.05$). Interestingly, occasional IgG1$^+$ and IgG4/7$^+$ epithelial cells were observed in the reproductive epithelium (Fig. 8C and D), indicative of IgG subtype transport across the epithelial lining.

In the case of the stallions that were actively shedding EAV in their semen at the conclusion of the study ($n = 2$), Ig-secreting (IgA$^+$, IgG1$^+$, IgG3/5$^+$, and IgG4/7$^+$) plasma cells were associated with the lymphoplasmacytic infiltration observed in the ampullae and bulbourethral glands (Fig. 7). A higher number of Ig-secreting plasma cells were observed in the ampullae of long-term carrier stallions than in those of the short-term carrier stallions and naive (control) stallions ($P < 0.05$), which demonstrates a certain degree of specificity of these effector cells to home within the main site of EAV persistence in the reproductive tract. Plasma cells in the ampullae and bulbourethral glands were also identified by transmission electron microscopy (TEM) (data not shown).
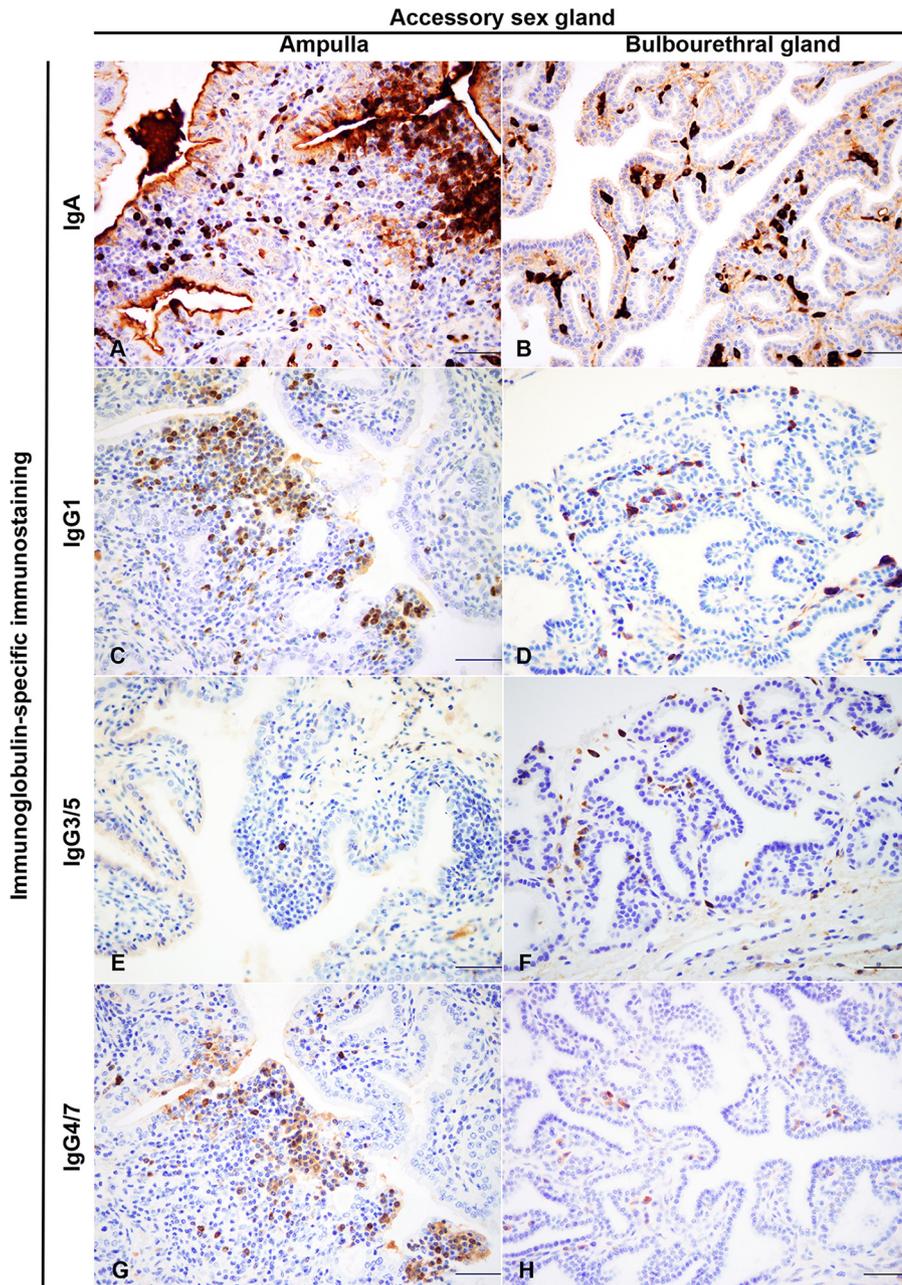
FIG 7 Immunohistochemical identification of plasma cells located in the accessory sex glands of stallions persistently infected with EAV. Specific immunoglobulin staining is indicated to the left, and the tissue type is indicated above each column. IgA+ plasma cells (A and B), IgG1+ plasma cells (C and D), IgG3/5+ plasma cells (E and F), and IgG4/7+ plasma cells (G and H) in ampullae (A, C, E, G) and bulbourethral glands (B, D, F, H) are shown. IHC with 3,3'-diaminobenzidine. Magnification, ×400. Bars, 50 μm.

DISCUSSION

In contrast to many viruses, EAV has evolved a complex mechanism that enables its long-term persistence (carrier state) in the stallion reproductive tract with no associated tissue damage, sperm abnormalities, or adverse effects on reproductive performance (13–15). Recently, it has been demonstrated that EAV persistence in the stallion reproductive tract occurs despite the induction of host systemic immune responses (e.g., strong neutralizing antibody response) and the induction of local inflammatory responses at the site of persistence (40). The strategy employed by EAV to successfully evade host immune surveillance has yet to be determined. Since the carrier stallion
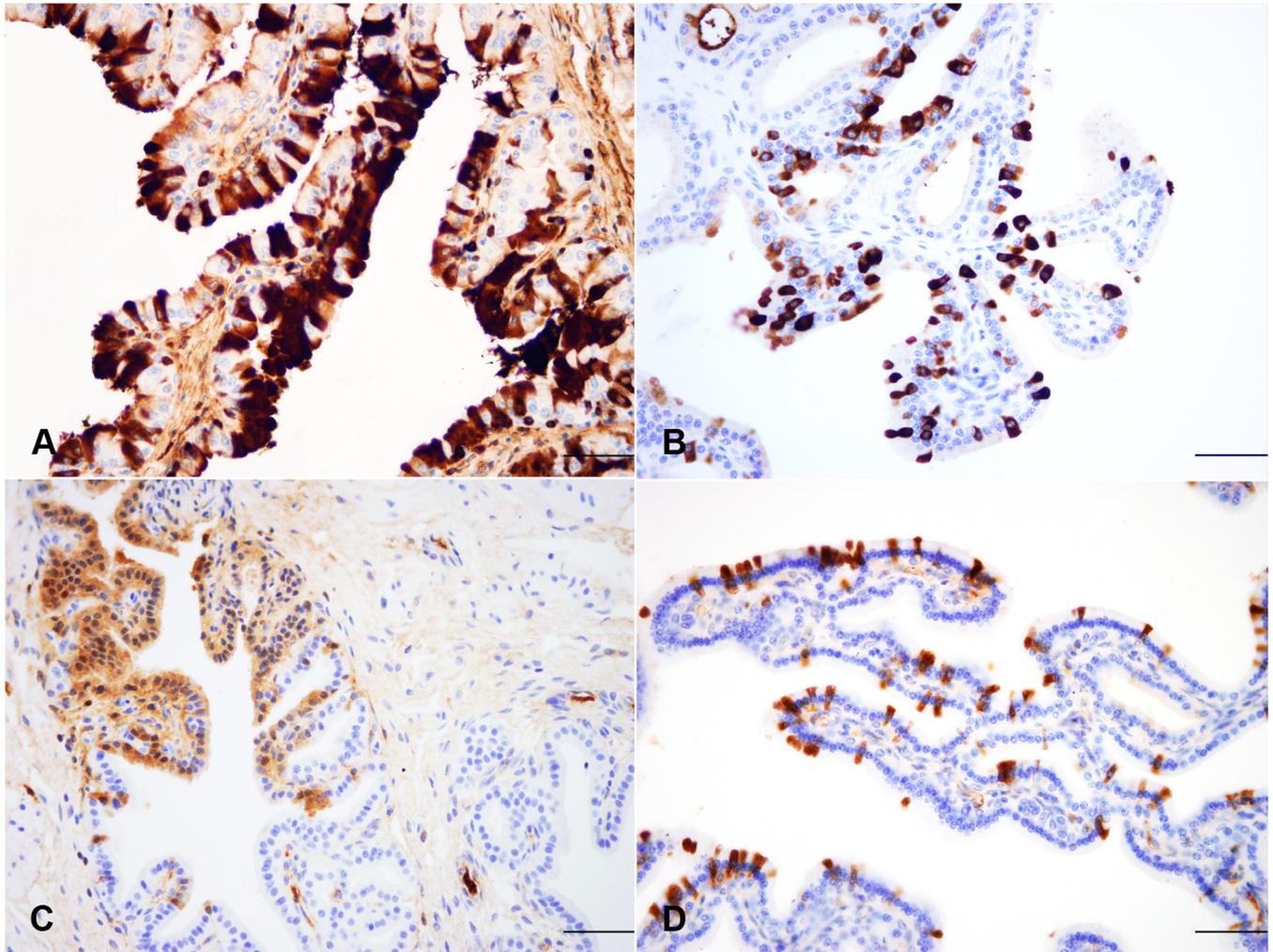
FIG 8 Immunohistochemical identification of immunoglobulin-positive epithelial cells in the accessory sex glands of stallions persistently infected with EAV. (A and B) IgA-specific epithelial immunostaining in ampulla (A) and vesicular gland (B); (C) IgG1-specific epithelial immunostaining in the vesicular gland; (D) IgG4/7-specific epithelial immunostaining in the vesicular gland. IHC with 3,3′-diaminobenzidine. Magnification, ×400. Bars, 50 μm.

plays a pivotal role in the epidemiology of EAV and constitutes the main challenge for its control and potential eradication (1, 5, 11, 12, 20), it is important to fully understand the underlying mechanisms involved in the establishment and maintenance of persistent infection in the stallion reproductive tract. Here, we investigated the antibody responses in short- and long-term persistently infected stallions following experimental EAV infection with a specific emphasis on the mucosal antibody response to aid in the understanding of the host immune factors involved in EAV persistence.

There are 11 immunoglobulin isotypes described in horse serum (IgA, IgD, IgE, IgG1 to IgG7, and IgM), and the role of these immunoglobulins during various viral infections has been the subject of extensive investigation (42–47). This study demonstrated that shortly after infection EAV induces a strong serum neutralizing antibody response comprised of a short-lived IgM response and a long-lasting IgG1 response. This response correlates with and likely leads to a reduction in the level of viremia and the clearance of viral infection from all body tissues, with the exception of the accessory sex glands of carrier stallions (40). Similar to the response observed following West Nile virus infection (47), the EAV-neutralizing antibody response was primarily mediated by IgG1 targeting the major viral structural proteins (GP5 and M) and nucleocapsid proteins, as previously described (38). Although IgG4/7 is the predominant serum IgG subtype in horses (43) and is involved in other antiviral responses, such as those against

equine herpesvirus 1 (48) or equine influenza virus (49), we were unable to detect EAV-specific systemic responses mediated by this or other antibody isotypes besides IgM and IgG1. This is indicative that other immunoglobulin isotypes either do not contribute to or play a minor role in systemic EAV-specific responses. However, a lack of detection of these responses may also be related to the diversity of these isotypes and technical limitations in identifying EAV-specific responses, as explained elsewhere (47). Most importantly, serum neutralizing antibodies are not effective in clearing persistent EAV infection in the reproductive tract. Even though the determinant factors are unknown, the cell types sustaining persistent EAV replication in the reproductive tract (40), an immunosuppressed tissue microenvironment (40), T lymphocyte dysfunction at the site of persistence, or even a limited accessibility of serum antibodies to reproductive tissues are all valid hypotheses that can explain the failure to clear persistent infection. These studies are in progress in U. B. R. Balasuriya's laboratory.

A significant finding from this study is that EAV infection induces a specific and diverse antibody isotype profile in seminal plasma and homing of plasma cells to the reproductive tract of infected stallions and, most importantly, that the virus has the ability to escape mucosal antibody responses, in spite of the variety of immunoglobulin isotypes involved in such a response. This study has demonstrated that, despite the presence of seminal plasma IgG1 and IgG4/7 with a neutralization capacity similar to that determined for serum IgG1 and the higher concentrations of IgG4/7 in seminal fluid derived from long-term persistently infected stallions, EAV continues to be shed in the semen of these stallions. Multiple factors may be related to the failure to neutralize EAV in reproductive tract tissues and seminal plasma, including but not limited to the emergence of novel phenotypic variants of the virus during persistent infection (escape mutants) (19, 26; Nam et al., unpublished), viral titers higher than the levels of specific immunoglobulins in seminal plasma available to neutralize the virus, other seminal plasma components that may interfere with or reduce immunoglobulin avidity and/or activation of the complement cascade (50–53), or extracellular vesicle (microvesicle)-mediated shedding of viral particles that allow escape from antibody surveillance, boosting viral propagation, as proposed for other viruses (54). Even though higher concentrations of EAV-specific seminal plasma IgG3/5 were observed in long-term carrier stallions, IgG3/5 seemed to bind to the EAV GP5 glycoprotein weakly and failed to neutralize EAV under *in vitro* conditions. Such findings may reflect the lower avidity of EAV-specific IgG3/5 for EAV proteins, a lack of significant affinity maturation in response to EAV, or the ability to activate the complement cascade (44). Similarly, secretory IgA in seminal plasma showed a lack of neutralizing activity, which could be related to the inability of this isotype to trigger complement activation through the classical pathway; rather, this isotype requires the mannose-binding lectin activation pathway instead (55).

In an attempt to identify the source of mucosal antibodies in the reproductive tract, we evaluated the homing of specific plasma cells to the accessory sex glands of infected stallions. The diversity in the plasma cell population observed in these tissues demonstrates the homing of these effector cells and that the local production of immunoglobulins is likely the source of secreted antibodies in seminal plasma, which contribute to the mucosal immune system in the reproductive tract. Interestingly, plasma cells have been proven to constitute another significant cell component of the inflammatory response previously described in long-term persistently infected stallions (40), and the persistence of these cells after interruption of viral shedding supports the fact that short-term carrier stallions maintain EAV-specific immunoglobulins in seminal plasma after viral clearance. Finally, immunohistochemical studies have also shown the transcytosis of IgA as well as other immunoglobulins of the IgG class, specifically, IgG1 and IgG4/7, through the reproductive epithelium. While IgA secretion through mucosal surfaces is associated with a transcytosis mechanism mediated by the polymeric immunoglobulin receptor (poly-IgR) in polarized epithelial cells (56), IgG secretion through epithelial surfaces has been associated with a specific Fc receptor in humans, namely, FcRn (57, 58). It is likely that this receptor is involved in this process, although

it has not been characterized in the horse thus far. Although the source of IgM in the seminal plasma of the accessory sex glands could not be determined since tissue collection was performed after the IgM response had waned, its response pattern was similar to the serological IgM response and IgM could have potentially gained access to the seminal plasma via the poly-Ig secretory pathway (56).

In conclusion, the study presented here demonstrates that although EAV induces both a serological antibody response and a mucosal antibody response in experimentally infected stallions, both responses are unable to clear viral persistence from reproductive tract tissues of long-term persistently infected stallions. While the persistence of EAV within reproductive tract lymphocytes may guarantee viral escape from serum neutralizing antibodies, seminal plasma antibody responses overall have been demonstrated to be only partially neutralizing (i.e., some IgG subtypes). We have also shown that the antibody isotype dynamics during EAV infection do not predict the occurrence of long-term EAV persistence in the stallion reproductive tract. This, along with the results of our previous studies in the area of EAV persistent infection in the stallion reproductive tract, demonstrates that a complex combination of host factors is involved in determining persistence or viral clearance from the reproductive tract.

MATERIALS AND METHODS

Cells and viruses. High-passage rabbit kidney 13 (RK-13 [KY]) cells (originally derived from ATCC CCL-37 cells; American Type Culture Collection) from passage levels 399 to 409 and equine pulmonary artery endothelial cells (EECs; University of California, Davis, Davis, CA) (59) were maintained as previously described (40). Tissue culture fluid (TCF) containing the virulent Bucyrus strain (VBS) of EAV (9) was used to generate antigen for the EAV-capture enzyme-linked immunosorbent assays (ELISAs) and Western immunoblotting. TCF containing the KY84 strain of EAV (from passage 1 in EECs; University of Kentucky, Lexington, KY) (37) was used for the experimental infection of stallions. TCF containing the commercial live-attenuated vaccine strain of EAV (strain MLV; ARVAC vaccine; Zoetis, Kalamazoo, MI) was used for the reference virus neutralization test (VNT) and modified microneutralization test as described below.

Stallions. A total of eight sexually mature stallions were included in the study. These were maintained at the Maine Chance Farm, University of Kentucky, Lexington, KY, and before initiation of the study were confirmed to be seronegative (titer < 1:4) for antibodies to EAV according to the World Organization for Animal Health (OIE) standardized VNT protocol (60) described below. The stallions were housed in individual stalls in an isolation facility for the duration of the study at the University of Kentucky in Lexington, KY. After experimental infection and monitoring for 726 dpi (see below), 2/8 stallions were classified as long-term carriers (duration of viral shedding in semen, >1 year), while 5/8 stallions were classified as short-term carriers (duration of viral shedding in semen, approximately ≤1 year). For the aforementioned reasons, the carrier status of a single stallion (stallion L143) could not be determined until postmortem examination, and the stallion was classified as a short-term carrier on the basis of the absence of infectious virus, viral antigen, and viral RNA from reproductive tract tissues derived from postmortem examination and minimal inflammation. A control group of naive stallions ($n =$ 4) remained unexposed and unvaccinated against EAV.

Animal care and ethics. This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (61). The Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky, Lexington, KY, approved this protocol (protocol number 2011-0888). Stallions were humanely euthanized by pentobarbital overdose following the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals, and all efforts were made to minimize suffering.

Experimental infection and clinical sample collection. Eight stallions (stallions L136 to L143) were intranasally inoculated in October 2011 with the KY84 strain of EAV (3.75×10^5 PFU per ml of TCF delivered in 5 ml Eagle's minimal essential medium [EMEM]) using a fenestrated catheter and monitored as previously described (40, 41, 62). Serum samples were sequentially collected in Vacutainer serum tubes (BD, Franklin Lakes, NJ) at −30 (prechallenge), 6, 8, 10, 14, 21, 28, 42, and 726 (preeuthanasia) dpi. Serum samples were heat inactivated at 56°C for 30 min, aliquoted, and stored at −20°C until tested. Semen samples were collected using an artificial vagina during the acute phase and after clinical recovery, specifically, at −30 (prechallenge), 1, 3, 5, 7, 9, 11, 13, 15, 23, 30, 44, 65, 86, 107, 128, 149, 170, 198, 226, 254, 282, 317, 345, 380, 407, 448, 462, 476, 497, 548, 701, and 726 (preeuthanasia) dpi, for the purpose of monitoring viral persistence. Semen samples were processed as previously described (17, 40). Seminal plasma samples were subjected to virus isolation and real-time reverse transcription-quantitative PCR to estimate the viral load and confirm the virus identity, as previously described (40). For immunoglobulin profiling, seminal plasma samples collected at −30 (prechallenge), 5, 11, 30, 107, 345, 548, and 726 dpi were tested.

Postmortem examination and tissue collection. Stallions were humanely euthanized by pentobarbital overdose following AVMA guidelines for the euthanasia of animals. Necropsy examination and tissue collection were performed at 2 years postinfection. For this study, the accessory sex glands (ampullae, vesicular glands, prostate, and bulbourethral glands) were collected. Tissues were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded following standard histological procedures.

TABLE 1 MAbs specific to the equine immunoglobulin isotypes used in this study

Specificity	Clone	Application[a]	Source (reference)
IgM	1-22	ELISA	Cornell University (42)
IgM	2B-63	IHC	Cornell University (42)
IgA	BVS2	ELISA, WB, isotype purification, IHC	Cornell University and University of Kentucky (63)
IgG1	CVS45	ELISA, WB, isotype purification, IHC	Cornell University (64)
IgG3/5	586	ELISA, WB, isotype purification	Cornell University (65)
IgG3/5	CVS38	IHC[b]	University of Kentucky (64)
IgG4/7	CVS39	ELISA, WB, isotype purification, IHC[b]	Cornell University and University of Kentucky (64)
IgG6	267	ELISA	Cornell University (65)

[a]ELISA, enzyme-linked immunosorbent assay; WB, Western immunoblotting; IHC, immunohistochemistry.
[b]Tissue culture fluid was utilized for IHC.

Additionally, tissues were fixed in 4% paraformaldehyde and 3.5% glutaraldehyde in 0.1 M Sorensen's buffer for 1.5 h and processed for transmission electron microscopy (TEM) examination.

Antibodies. A panel of monoclonal antibodies to equine immunoglobulin isotypes were used for EAV-capture ELISA, immunohistochemistry (IHC), isotype purification, and Western immunoblotting, as described below (Table 1) (42, 63–65). In addition, a MAb specific to EAV GP5 (MAb 6D10) (28) was purified from ascitic fluid (following a conditioning step with ascites conditioning reagent [Thermo Fisher Scientific, Waltham, MA]) using a Melon gel IgG purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions and biotinylated using an EZ-Link sulfo-NHS biotinylation kit (Thermo Fisher Scientific) following the manufacturer's recommendations. Biotin incorporation was evaluated by a 4′-hydroxyazobenzene-2-carboxylic acid assay as recommended by the manufacturer, and the specificity of biotinylated MAb 6D10 was further evaluated by Western immunoblotting and ELISA. Immunohistochemical staining was performed using a Bond Polymer Refine Detection kit (Leica Biosystems, Buffalo Grove, IL) as described below. In addition, a goat polyclonal antibody specific to equine IgM (SeraCare Life Sciences, Milford, MA) was used for the dot blot test. Secondary antibodies conjugated to horseradish peroxidase (HRP; goat anti-mouse IgG [H+L]-HRP and goat anti-rabbit IgG [H+L]-HRP [SouthernBiotech, Birmingham, AL]) were used for Western immunoblotting.

Antigen production for ELISA. The prototype VBS strain of EAV was propagated once in EECs to prepare high-titer working stocks as previously described (28, 66). Briefly, EECs were plated in tripledecker flasks and infected with infectious TCF containing the VBS strain of EAV. After a 90 to 100% cytopathic effect (CPE) was reached, infected flasks were frozen-thawed once ($-80°C$), and cell lysates were clarified by centrifugation ($3,500 \times g$) at 4°C for 15 min and filtered through a 0.45-μm-pore-size filter unit (Nalgene; Thermo Fisher Scientific). Clarified TCF was subsequently ultracentrifuged (Beckman Coulter, Miami, FL) at $121,600 \times g$ through a 20% sucrose cushion in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) at 4°C for 4 h to pellet the virus. Partially purified preparations were resuspended in a minimal volume of $1\times$ phosphate-buffered saline (PBS; pH 7.4; Gibco) and titrated by plaque assay on EECs, and total protein was quantified using a spectrophotometer (absorbance at 280 nm) (BioTek Synergy H1 multimode microplate reader; BioTek Instruments, Inc., Winooski, VT) and frozen at $-80°C$ until it was used. Viral stocks contained a viral titer of 1×10^9 PFU/ml.

Pooled negative serum and seminal plasma samples. A pooled negative serum sample was prepared by mixing equivalent amounts of individual serum samples from EAV-seronegative stallions ($n = 12$; stallions L136 to L143 [prechallenge], N105, N121, O103, and O113). Similarly, a pooled negative seminal plasma sample was prepared from EAV-seronegative stallions ($n = 11$; stallions L136 to L142 [prechallenge], S-6432, S-6435, S-6465, and S-6519). These pooled samples were used as assay controls in isotyping ELISAs.

Virus neutralization test. Neutralizing antibodies in serum were evaluated by VNT using the standardized protocol described in the OIE *Manual of Diagnostics and Vaccines for Terrestrial Animals* (60) and a virus working suspension containing 100 50% tissue culture infective doses (TCID$_{50}$) of the commercial live-attenuated vaccine strain of EAV (strain MLV) per 25 μl in $1\times$ EMEM containing 10% guinea pig complement (Rockland Immunochemicals, Gilbertsville, PA). The use of the MLV strain of EAV was based on the existence of a single viral serotype broadly neutralized by polyclonal equine antiserum and on its standardized use for EAV serological testing, as indicated in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (60). The neutralizing antibody titer was recorded as the reciprocal of the highest serum dilution that provided at least 75% neutralization of the reference virus.

EAV-specific isotyping ELISAs. To determine the EAV-specific immunoglobulin isotype profiles in serum and seminal plasma, a panel of EAV-capture ELISAs was developed by using the MAbs listed in Table 1 and as previously reported (47). Briefly, 96-well enzyme immunoassay/radioimmunoassay polystyrene high-binding plates (Corning) were coated with 100 μl/well containing 4 μg/ml of the corresponding MAb (anti-equine immunoglobulins; Table 1) in coating buffer (0.2 M sodium carbonatebicarbonate buffer, pH 9.4 [BupH carbonate-bicarbonate buffer packs; Thermo Fisher Scientific]) and incubated overnight at 4°C. The coated plates were washed 5 times with an ELISA wash buffer ($1\times$ PBS containing 0.05% Tween 20 [Bio-Rad, Hercules, CA]) using a multiwell washer (Nunc-Immuno; Thermo Fisher Scientific) and blocked with 5% skim milk in $1\times$ PBS containing 0.05% Tween 20 (200 μl/well) for 1 h at room temperature. Following a washing step as described above, each serum sample (diluted

1:100 in blocking buffer) or seminal plasma sample (undiluted) was added in quadruplicate (50 μl/well), and the plate was incubated at 37°C for 1 h. The plates were subsequently washed as described above, and either partially purified EAV VBS (positive capture) or a gradient-purified protein preparation derived from mock-infected EECs (negative capture) at a concentration of 30 μg/ml of protein was added to each of 2 wells (50 μl/well), respectively. The plates were incubated with the respective antigen overnight at 4°C and washed as described above, followed by incubation with a biotinylated EAV anti-GP5 MAb (MAb 6D10, diluted 1:100 in blocking buffer) for 1 h at 37°C (50 μl/well). After a washing step, the plates were subsequently incubated with a streptavidin-HRP conjugate (50 μl/well Pierce high-sensitivity streptavidin-HRP [Thermo Fisher Scientific] diluted 1:4,000 in blocking buffer) for 1 h at 37°C. 3,3',5,5'-Tetramethylbenzidine (TMB; 50 μl/well; 1 component; SureBlue; KPL, Gaithersburg, MD) was used as the substrate, and the reaction was allowed to develop for 10 to 15 min for serum samples or 7 min for seminal plasma samples at room temperature in the dark and stopped by adding 50 μl/well of a 0.18 M sulfuric acid solution. The plates were read at a wavelength of 450 nm using a BioTek Synergy H1 multimode microplate reader (BioTek Instruments, Inc.). A pooled negative serum sample and a seminal plasma sample were included as negative controls on every plate. Average optical density (OD) values were calculated after subtraction of the blank for the positive- and negative-capture wells. A positive/negative ratio (PNR) was then calculated for each sample, as follows: PNR = (mean OD for positive capture of the test sample/mean OD for positive capture of the pooled negative control sample). A PNR cutoff value was calculated as follows: PNR cutoff = (mean PNR for prechallenge samples) + 2 × SD, where SD is the standard deviation of the PNR for prechallenge samples. A PNR of ≥2 was considered a positive response for all ELISAs except the IgM-capture ELISA performed on seminal plasma, which was determined to have a PNR cutoff value of 3.

Isotype purification from serum and seminal plasma. Serum IgG1 and seminal plasma IgA, IgG1, IgG3/5, and IgG4/7 from serum and seminal plasma samples (obtained before challenge and at 726 dpi) pooled from short-term carrier stallions (stallions L137, L138, L139, L141, L142, and L143) and long-term carrier stallions (stallions L136 and L140) were purified using an affinity column coupled to each MAb to equine-specific immunoglobulin isotypes (AminoLink Plus Micro immobilization kit [Thermo Fisher Scientific]). Briefly, 200 μl of MAb (1 mg/ml) was coupled to the resin support overnight at 4°C by end-over-end mixing, as recommended by the manufacturer. After MAb coupling, the remaining active binding sites on the resin were blocked with a quenching buffer and sodium cyanoborohydride solution, as instructed by the manufacturer. For affinity purification, 300 μl of the sample (undiluted pooled serum or seminal plasma previously clarified by centrifugation at 3,000 × g for 15 min at 4°C) was added onto the resin and allowed to bind for 2 h at room temperature with end-over-end mixing. The column was subsequently washed as instructed by the manufacturer, and bound immunoglobulins were eluted two times using 100 μl of elution buffer (pH 2.8). Eluates were immediately neutralized by adding 5 μl of 1 M Tris buffer (pH 9.0) per 100 μl of eluate, and purified immunoglobulins were quantified by determination of the OD at 280 nm using a spectrophotometer (NanoDrop 2000 spectrophotometer; Thermo Fisher Scientific). Eluted immunoglobulins were evaluated by dot blot testing. Briefly, 5 μl of each eluate was dotted onto a nitrocellulose membrane (Bio-Rad) and air dried. The membrane was subsequently blocked in 5% skim milk in 1× Tris-buffered saline containing 0.05% Tween 20 (TBS-T; pH 7.6) and incubated with MAbs to equine immunoglobulins (1 μg/ml in blocking buffer) overnight at 4°C. After extensive washing with TBS-T, the dotted membranes were incubated with a secondary antibody (goat anti-mouse IgG conjugated with HRP diluted 1:5,000 in blocking solution) for 1 h at room temperature and developed as indicated below (see "Western immunoblotting" below). To verify the specificity of the eluates, membranes were incubated with goat anti-equine IgM conjugated with HRP (diluted 1:1,000 in blocking solution) for 1 h at room temperature and subsequently developed as indicated below.

Western immunoblotting. Western immunoblotting was performed to determine the specificity of the different EAV-specific isotypes in serum and seminal plasma for viral structural proteins. Briefly, gradient-purified EAV strain VBS (28, 29) was resuspended in 5× Pierce lane marker reducing sample buffer containing 0.3 M Tris-HCl, 5% SDS, 50% glycerol, and 100 mM dithiothreitol (DTT) (Thermo Fisher Scientific) and then subjected to denaturing SDS-PAGE on a 12% resolving gel and 4% stacking gel. Subsequently, viral proteins subjected to PAGE were wet transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 1 h at 4°C. Transferred PVDF membranes were briefly washed in TBS-T and blocked with 5% skim milk in TBS-T overnight at 4°C. Blocked PVDF membranes were briefly rinsed in TBS-T and loaded onto a miniblotter (20SL; Immunetics, Cambridge, MA). Pooled seminal plasma samples (obtained before challenge and at 726 dpi) were added undiluted (350 μl/lane), while pooled serum samples (obtained before challenge and at 726 dpi) were diluted 1:20 in blocking buffer. Membranes were incubated for 2 h at room temperature with gentle rocking, washed 4 times for 5 min each time with TBS-T, and incubated with MAbs to selected equine immunoglobulin isotypes (diluted to 1 μg/ml in blocking buffer) for 1 h at room temperature with gentle rocking. After subsequent washes, the membranes were incubated with goat anti-mouse IgG (diluted 1:5,000 in blocking buffer) for 1 h at room temperature and developed using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific), as indicated by the manufacturer. The developed membranes were imaged using an Azure c300 digital imager system (Azure Biosystems, Dublin, CA). In addition, one membrane lane was probed with an antibody cocktail (1 μg/ml each antibody in blocking buffer) specific to the EAV GP5 (MAb 6D10), M (rabbit polyclonal antibody 7888), and N (MAb 3E2) proteins (28–30, 67, 68). A secondary antibody cocktail containing goat anti-mouse and anti-rabbit IgG (diluted 1:5,000 in blocking buffer) was then used.

Modified microneutralization test to assess the neutralizing activity of purified serum IgG1 and purified seminal plasma IgA, IgG1, IgG3/5, and IgG4/7. The neutralizing activity of purified immu-

TABLE 2 Immunohistochemical scoring system used to assess specific immunoglobulin-producing plasma cell infiltration in the accessory sex glands

Score	No. of immunopositive plasma cells[a]
0	None
1	<5
2	5–40
3	41–80
4	>80

[a]Cumulative number of immunopositive plasma cells in five microscopic fields with a total magnification of $\times400$.

noglobulin isotypes derived from serum (IgG1) and seminal plasma (IgA, IgG1, IgG3/5, and IgG4/7) was evaluated in a modified microneutralization test. Briefly, serial 2-fold dilutions (1:4 to 1:512) of each purified immunoglobulin preparation were prepared in 10 μl of EMEM (Cellgro) supplemented with 10% ferritin-supplemented calf serum (HyClone Laboratories, Inc.) and added to triplicate wells in a 384-well plate (Corning). Ten microliters of a working suspension of the commercial live-attenuated vaccine strain of EAV (strain MLV; ARVAC vaccine; Zoetis) containing 50 $TCID_{50}$/10 μl in EMEM supplemented with 10% guinea pig complement (Rockland Immunochemicals) was added to each of duplicate wells, and the plates were incubated for 1 h at 37°C in 5% CO_2. The third replicate was used as a cytotoxicity control. Finally, 40 μl of a high-passage-number RK-13 (KY) cell suspension was added to each well, and the plates were incubated at 37°C in 5% CO_2 for 72 h. The neutralizing antibody titer was recorded as the reciprocal of the highest serum dilution that provided at least 75% neutralization of the reference virus. Neutralizing antibody titers were subsequently converted into the minimal concentration of specific isotype immunoglobulin (micrograms per milliliter) necessary for virus neutralization.

Histopathology and TEM. Sections of formalin-fixed paraffin-embedded (FFPE) tissues (5 μm) were stained with hematoxylin and eosin (H&E) following a standard procedure prior to histological evaluation. Tissue sections were scrutinized by an experienced veterinary pathologist who was blind to the carrier status of the stallions, and a morphological diagnosis was provided. Inflammatory lesions were scored as previously described by Carossino et al. (40). For TEM, samples were fixed as indicated above, postfixed in 1% osmium tetroxide (OsO_4) for 1.5 h at 4°C, embedded in Eponate12 resin (Ted Pella, Inc., Redding, CA), and polymerized for 48 h at 60°C. Ultrathin sections were obtained using a Reichert Ultracut E ultramicrotome (Leica Biosystems, Buffalo Grove, IL), collected on copper grids, and counterstained with 4% uranyl acetate solution and Reynold's lead citrate. Images were obtained using a Philips Tecnai BioTwin 12 transmission electron microscope (FEI, Hillsboro, OR).

Immunohistochemistry. Sections of FFPE tissues (5 μm) were mounted on positively charged Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and dried overnight at 37°C. For IgA, IgM, IgG1, IgG3/5, and IgG4/7 immunostaining, deparaffinization was performed as previously described (40). Heat-induced epitope retrieval (HIER) was performed using a modified citrate-based ready-to-use solution (pH 6.1; Dako, Carpinteria, CA) at 96°C for 30 min. The slides were allowed to cool for 20 min and washed three times in 1$\times$ PBS. Immunostaining was performed using a Bond Polymer Refine Detection kit (Leica Biosystems), and tissue sections were counterstained and mounted as previously described (40). Palatine tonsil sections were used as both positive and negative (without primary antibody incubation) controls. The cumulative number of immunohistochemistry-positive plasma cells in five high-magnification ($\times400$) fields was quantified, and a score was assigned, as indicated in Table 2.

Statistical analysis. Data distribution, descriptive statistics, plots, and statistical tests were generated using JMP10 statistical analysis software (SAS, Cary, NC). Normality and equal variances were assessed by the Shapiro-Wilk and Hartley's test, respectively. Neutralizing antibody data lacked normality; consequently, nonparametric tests were used for statistical analysis (Kruskal-Wallis test and Wilcoxon signed-rank test). Analysis of variance (ANOVA) was used to compare ELISA PNRs between short- and long-term carrier stallions and between data points (days postinfection). For the latter, Dunnett's test was used for multiple comparisons using the baseline PNRs (prechallenge) as controls. Data sets that did not comply with the assumption of equal variance (serum and seminal IgG3/5 and IgG4/7 PNRs between short- and long-term carrier stallions) were analyzed using the Welch test. Correlation analysis between serum neutralizing antibody titers and the serum IgG1 PNR, as well as between serum neutralizing antibody and viremia titers, was performed by Spearman's rank correlation method. Statistical significance was set at a P value of <0.05 for all tests performed.

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